HHS Public Access

Author manuscript

Mech Dev. Author manuscript; available in PMC 2016 May 01.

Published in final edited form as:

Mech Dev. 2015 May ; 136: 73–86. doi:10.1016/j.mod.2015.01.002.

Expansion of specialized epidermis induced by hormonal state and mechanical strain

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Abstract

In mammals, some sites of specialized skin such as the palms, soles, and lips grow proportionally with the animal. However, other types of specialized skin such as the nipple and anal/genital region are dramatically altered with changes of reproductive status. The specific cell types that mediate the growth of these sites have not been identified. In the mouse, we observed a dramatic expansion of the specialized epidermis of the nipple, coupled to changes in connective tissue and hair shaft density, which we designate as areola formation. During this process thymidine analog uptake was elevated in the epidermis and hair follicles. Although there were no changes in connective tissue cell proliferation, we did observe an altered expression of extracellular matrix genes. In addition, the fibroblasts of the virgin nipple areola and region showed increased transcript and protein levels for estrogen, progesterone, relaxin, and oxytocin relative to those of ventral skin. To determine the role of pregnancy, lactation hormonal milieu, and localized mechanical strain on areola formation, we created models that separated these stimuli and evaluated changes in gross structure, proliferation and protein expression. While modest increases of epidermal proliferation and remodeling of connective tissue occurred as a result of individual stimuli, areola formation required exposure to pregnancy hormones, as well as mechanical strain.

Keywords

Nipple; areola; keratins; lactation; pregnancy; hormones; mechanical strain

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CONFLICT OF INTEREST

The authors state no conflict of interest.

INTRODUCTION

Regions of specialized epidermis are observed across all vertebrates. These areas are vital in complex behaviors such as sensation, locomotion, and reproduction. The epidermis of these regions is characterized by: 1) reduced numbers of hair, feathers or scales and often coupled with the presence of unique appendages such as specialized glands; 2) distinct patterns of stratification; 3) expression of cytoskeletal proteins that are not present in the epidermis of the trunk (Koyama et al., 2013). In the human, regions of specialized epidermis include the lips, eyelids, the palmar and plantar skin, as well as portions of external genitalia. Rodents possess these examples and additional specialized epidermal regions including the vibrissae-bearing muzzle, ear, and tail (Mahler et al., 2004; Schweizer, 1993). Another locale of specialized epidermis is the nipple, a hairless region of the epidermis used to deliver milk found in placental mammals (Eastwood et al., 2007; Mahler et al., 2004).

The murine nipple has a thickened epidermis that possesses a moderate alteration in both the expression of the differentiation markers found in the trunk skin, as well as keratins K2e and K6 (Abdalkhani et al., 2002; Foley et al., 2001; Mahler et al., 2004). The development and maintenance of specialized epidermis is dependent upon inductive signals from the underlying dermal connective tissue (Dhouailly et al., 1998). The connective tissue that underlies the nipple in the adult appears to be derived from the primary mammary mesenchyme, which directs its formation at E17 in the mouse (Dunbar et al., 1999).

During murine postnatal life, some examples of specialized epidermis, such as the tail and ear, appear to proportionally expand with the growth of the organism, yet do not appear to overtly alter their cellular structure between neonatal and adult life. However, this static morphology probably does not typify most specialized epidermal sites. The formation of a thick callus on palmar and plantar sites in response to pressure indicates the capacity to alter epidermal proliferation and differentiation programs in response to weight bearing strain (Menz et al., 2007). The human nipple and areola both increase in diameter 3-fold during puberty (Rohn, 1987). During pregnancy and lactation, they further increase in diameter, and the epidermis becomes more pigmented (Neifert et al., 1990; Rohn, 1989). During pregnancy and lactation, the nipple of both rats and mice lengthens and increases in diameter ~3-fold (Toyoshima et al., 1998a; Toyoshima et al., 1998b); however, in these species the presence of an areola has not been noted.

In this manuscript, we investigated changes that occurred in the murine nipple over pregnancy, lactation, and weaning. We found that a relatively hairless ridge of specialized epidermis forms around the base of the nipple during the first lactation. We concluded that this region serves as the areola.

RESULTS

2.1 Formation of the murine areola

The virgin nipple is a very small structure less than 0.5mm in diameter that sits in a depression or cleft, its apex is minimally raised above the surrounding skin, and is obscured by hair. During pregnancy and lactation, the murine nipple lengthens 3-fold (Toyoshima et

al., 1998a; Toyoshima et al., 1998b) but the structure does not become visible (Fig.1A). However, 2 days after parturition, the nipple was surrounded by a ridge of relatively glabrous skin that expanded maximally by 3-weeks postparturition (Fig. 1A and B). One year post-lactation, a patch of relatively hairless skin around the nipple was retained (Fig. 1A).

2.2 Epidermis, dermis and hair follicles in the areola region

We next examined the histology of nipples and surrounding skin of virgin, late pregnant (18 to 18.5 days post coitus), early lactating (2-days), mid-lactating (1-week) and post-lactating (6 months) mice. In the virgin, the thickened epidermis terminates abruptly at the midpoint of the cleft that surrounds the base of the nipple. During pregnancy, the nipple proper broadens, and the thickened epidermis begins to expand within the cleft. After 2 days of lactation, the region of thickened epidermis expanded 1 to 1.5mm around the nipple, and this was further extended to 3.5 mm beyond the structure in the 1-week lactating samples. Thickened epidermis outside of the nipple was not present 6 months post-lactation. The epidermis of the virgin nipple was highly undulated into deep pegs particularly in the cleft region, where as in the pregnant and lactating samples these pegs recede and cracks or wrinkles form in the outer layer of the nipple and at its base. Note that in the post lactating sample the cleft a the base of the nipple disappeared and a narrow epithelial strand was present. We used histomorphometry to illustrate these changes in the epidermis by measuring both the outer epidermal surface and the epidermal/dermal junction between the hair follicles on either side of the nipple. The ratio of the outer to inner length was ~0.6 in the virgin, increased to ~1 in the pregnant and lactating samples, and then decreased in the post lactation samples (Fig. S1)

Various differentiation markers present in the nipple were found in the thickened epidermis surrounding the structure. As shown in Figure 2, filaggrin and K6 expression was much more apparent in the skin surrounding the nipple in the 2 day lactating samples, indicating expanded suprabasal epidermal layers (Fig. 2A). K2e immunoreactivity, which is normally patchy and restricted to the virgin nipple, was surprisingly present in the surrounding thin epidermis in late pregnant nipples (Fig. 2A). However, antibody staining diminished in the cytoplasm and appeared to localize mainly to the nuclei in the lactating nipples. In 6-month post lactation samples, patchy K2e and K6 immunoreactivity remained in the surrounding epidermis, but this region was not thickened (Fig. 2A). Short term BrdU incorporation and antibody labeling was performed to identify epidermal cells in S-phase of the cell cycle. The nipple and the areola (when present) had high BrdU incorporation as compared to the surrounding epidermis (Fig. 2B,C,D). In the pregnant and 2-day lactation samples, the nipple, areola, and epidermis had increased BrdU incorporation relative to other stages. At one-week lactation; however, BrdU antibody labeling in the nipple and areola was diminished (Fig. 2B&D). Compared to the basal level in the virgin, the post-lactation mice exhibited significantly less incorporation of the thymidine analog in the nipple (Fig. 2B&D). We also used terminal deoxynucleotidyl transferase (mediated deoxyuridinetriphosphate nick end labeling (TUNEL) to evaluate apoptosis in the various samples. Labeled cells were occasionally found in suprabasal layers above pegs or the cleft in the virgin or within creases of pregnant and early lactating nipples (Fig. S2). Taken together, the nipple lengthens during

pregnancy and lactation but additionally there is a formation of a ridge of thickened, and highly proliferative epidermis that expressed nipple keratin markers, that appears to serve as a functional areola.

Next, connective tissue was evaluated using Herovici's stain. The virgin nipple and surrounding skin had high levels of mature collagen fibers (red stain in Fig. 3A), whereas the lactating nipple and surrounding region contained primarily immature collagen fibers (blue stain), and the late pregnant nipple exhibited a mixture of smooth muscle actin (SMA) antibody labeled thin groupings of cells along the lactiferous duct within the nipple and between the mammary fat pad and dermis of virgin and late pregnant samples (Fig. 3A). However, after 2-days of lactation and onward, thick bands of SMA antibody-labeled cells with smooth muscle morphology extended under the entire region of thickened epidermis (Fig. 3A). There was minimal BrdU incorporation into the smooth muscle appearing cells or fibroblasts of the nipple or areola region during lactation and pregnancy (Fig. S3A). However BrdU incorporation occurred in the underlying connective tissue in the cleft region of late pregnant samples, in what appeared to be cells associated with capillaries (Fig. S3A).

We used qRTPCR to probe changes in extracellular matrix production in the nipple samples, and *collagen I* α 2 mRNA levels decreased during pregnancy and lactation, whereas *collagen III* α 1 and *Fibronectin* was increased at these stages (Fig. 3B). We also used picro-sirius red stain and circular polarized microscopy to analyze the collagen I and collagen III (Ren et al., 2013) expression. Focusing on the virgin and 2-day lactating samples, we observed an increase in the bright yellow patches that correspond to Collagen III protein in the nipples of 2-day lactating samples (Fig. 3C).

Given that hair shafts were diminished in the region around the lactating nipple, we examined the histology of the hair follicles in this region with ALX4 immunolabeling (upper row of Fig. 4A) of cells within the dermal papillae, and Masson’s trichrome stain (lower row of Fig. 4A). As shown in Figure 4A, hair follicles in close proximity to the nipple maintained their dermal papilla in all stages of the reproductive cycle (Rendl et al., 2008). We extended our analysis of the hair cycle of hair follicles that border the nipple in lactating dams at 5, 7,11,14, 16,19, 21 days post-delivery and 3 days post-nursing (pups were weaned on post-natal day 21). The hair follicles immediately around the nipple were in telogen in the late pregnant samples, (Fig. 4A). In early lactation samples, most of these hair follicles were also in telogen, whereas in 7 to 21-day samples many telogen follicles remained, but the fraction of those in anagen tended to increase (additional time points not shown). However, this was highly variable probably reflecting the frequency of nursing by the litter, the number of pups, and the amount of suckling on the individual nipples. Three days after weaning, most hair follicles around the nipple were in mid-anagen. Hair follicle stage on ventral skin distal from the nipples tended to reflect that of the area immediately surrounding the structure; nonetheless, hair follicles on the dorsal surface entered anagen at parturition (Fig. S4A and B), as has been previously reported by Chuong and colleagues (Plikus and Chuong, 2008; Plikus et al., 2008).

To evaluate sebaceous gland size, we used whole mounts of Herxheimer’s-stained skin to measure the area of the glands associated with the first four rows of hair follicles around the

nipple at various times of pregnancy and lactation (Fig. 4B). During pregnancy and lactation, the sebaceous gland was much more intensely stained and there appeared to be a modest but progressive increase in its area such that by 3-weeks of nursing there was 1.5-fold increase (Fig. 4C). BrdU incorporation was also assessed in the hair follicles proximal to the nipple (Fig. S3B). During pregnancy and lactation, the hair follicle infundibulum thickened, coupled with increased BrdU incorporation.

2.3 Hormone receptor expression in nipple fibroblasts

The expansion of connective tissue in the nipple during pregnancy and lactation suggest that the fibroblasts in this region may be sensitive to reproductive hormones. We examined the expression of pregnancy and lactation related hormone receptor transcripts, including estrogen receptor α (Esr1), oxytocin receptor (Oxtr), progesterone receptor (Pgr), prolactin receptor (Prlr) and relaxin receptor (Rxfp1) from carefully dissected virgin nipple, and ventral skin pieces of WT mice. Using qRTPCR analysis, we found that *Esr1, Oxtr, Pgr* and *Rxfp1* but not *Prlr* mRNA were significantly up-regulated (Fig5 A). The virgin nipple is a very small structure, so to obtain large pieces of nipple-like tissue, as well as high numbers of nipple fibroblasts for this evaluation, we used the ventral skin from the virgin keratin-14 promoter driven PTHrP (K14-PTHrP) transgenic mouse, in which this entire surface is converted to nipple skin (Foley et al., 2001). As shown in Figures 5A, the ventral skin of the virgin K14-PTHrP mouse had elevated levels of hormone receptor transcripts similar to the nipples of wild type. To confirm the elevated hormone receptor transcripts occurred in fibroblasts and not other cells of the nipple, we used antibodies to platelet-derived growth factor receptor (Pdgfrα), a marker for dermal fibroblasts (Collins et al., 2011), to sort these cells from virgin wild-type (WT) ventral skin or K14-PTHrP ventral skin. As shown in Figure 5 B, qRTPCR analysis indicated that Pdgfra+/K14-PTHrP fibroblasts had significantly increased *Esr1, Oxtr, Pgr* and *Rxfp1* but not *Prlr* transcript levels.

Next, protein levels of the hormone receptors were analyzed by immunofluorescence to obtain evidence of differential expression of the receptors, as well as cellular location. Western blotting of extracts from cultured fibroblasts from WT and K14-PTHrP mice confirmed expression data. In virgin mice, Esr1, PgR, Rxfp1, and Oxtr antibody labeling was more intense in nipple fibroblasts as compared to ventral skin regions (Fig. 6A–D). Morphologically, these hormone receptor antibody-labeled fibroblasts were small and sporadic in the virgin ventral dermis. On the other hand, hormone receptor positivenipple fibroblasts, were larger spindle-shaped, densely packed and aligned with each other. Some nuclear Esr1 labeling was observed in the nipple fibroblasts, but not in those from the ventral dermis. Nuclear progesterone receptor or nuclear phospho-cAMP responsive binding protein (phospho-CREB), the mediator of relaxin signaling, labeling was not present in either virgin nipple or ventral dermal fibroblasts. In late pregnant WT mice, there were more Esr1, PgR, Rxfp1, and Oxtr antibody labeled fibroblasts in the nipple than the ventral skin (Fig. 6A–D). Furthermore, the number of nuclear Esr1, nuclear PgR labeled cells was substantially increased in nipple fibroblasts of pregnant animals, as compared to the ventral dermis (not shown). Strong nuclear phospho-CREB was detected in the pregnant nipple fibroblasts, but was absent in ventral dermal fibroblasts. Western blotting indicated Esr1, PgR and Oxtr bands were increased in cultured fibroblasts from virgin K14-PTHrP ventral

skin, as compared to those from the ventral skin of WT littermates, but Rxfp1 labeling was not increased (Fig 6E).

2.4 Requirements for areola formation

In order to evaluate the impact of hormones and mechanical strain on the formation of the areola, we created models with conditions wherein the nipple or ventral skin of the K14-PTHrP mouse was exposed to high levels of hormones associated with pregnancy and/or lactation, but was not subject to localized mechanical strain. If pups were removed from the mother immediately after parturition, the areola did not form (Fig. 7A). However if pups were returned within ~40 hours, the areola formed (Fig. 7B). When pups were introduced to a dam after 72 hours, the pups would attempt to suckle, but the areola would not form (Fig. 7C), presumably from the inability of the dam to produce milk, which limited suckling. Using a model where certain nipples were nursed while others were not, the areola formed on those that had been suckled (Fig. 7D). In addition, we used the ventral skin of the K14-PTHrP mouse to evaluate nipple-like skin that is not nursed, for testing the impact of hormones relative to mechanical strain. The gross appearance of the transgenic skin did not change markedly during pregnancy and lactation (Fig. 7E). Finally we exposed the nipple or nipple-like skin to localized mechanical strain under conditions where pregnancy and lactation hormones were not elevated. To achieve this we used virgins that were suckled over a 48-hour period by multiple squads of pups. We observed extensive suckling and the virgin's nipples often became red and inflamed, but an areola did not form (Fig. 7F). Also, we used an artificial nursing method to simulate the mechanical strain on the K14-PTHrP skin (Eastwood et al., 2007) (Fig. S5), which again produced no apparent changes. The use of multiple models suggested that pregnancy and lactation hormones alone, or mechanical strain by itself, are not sufficient to trigger areola formation.

We evaluated nipple tissue with stains and antibodies used in Figures 1 and 2. In nursed virgin nipples, K2e staining was decreased in the cytoplasm, but otherwise nipple epidermal markers were not expanded into the areola region. Also in the suckled virgin nipple BrdU incorporation occurred in the epidermis of the cleft (Fig. 7G), but extensive uptake was also observed in the connective tissue cells of this region, which normally does not occur during lactation (Fig. 7G). In the models where nipples were not suckled after pregnancy there was a lengthening and broadening of the structure (Fig. 7G), smooth muscle expanded (Fig. 7G) commensurate with changes that occur in pregnant nipples. Also, BrdU incorporation in the epidermis occurred in the cleft of the non-nursed nipples in the lactating mouse (Fig. 7G). In the K14-PTHrP ventral skin samples, staining for immature collagen and epidermal proliferation increased during late pregnancy similar to the nipple, otherwise many of the changes associated with lactating nipples were not apparent (Fig. S5). However in transgenic ventral skin that had been artificially nursed over 48 hours (Fig. S5), K2e staining was decreased in the cytoplasm as observed in the nursed virgin and lactating nipple samples. In conclusion, mechanical strain alone can induce epidermal and dermal proliferation, as well as alter the keratin cytoskeleton of the epidermis, pregnancy hormones are likely to stimulate the connective tissue changes that underlie much of the nipple growth and areola formation, and lactation hormones have relatively minimal effect.

DISCUSSION

We propose the following model to explain the growth of the murine nipple and formation of a functional areola (illustrated in Fig 8) that involves a coordinated cellular response by the epidermis, dermis, and hair follicles. In virgin nipple, the smooth outer layer of thickened epidermis stops at the base of the cleft, but the basal epidermis is marked by large invaginations or pegs and the junction of nipple and skin represents the most prominent infold. At 2-days after parturition, the nipple approaches its maximal length, epidermal creases and wrinkles form and the epidermis thickens around the nipple forming the areola. At this time, the thickened epidermis and nipple markers of K6 and K2e expand outside of the cleft. TUNEL labeling is found in the outer suprabasal cells in the creases, suggesting programed cell death may underlie the formation of these structures, providing a means of "unzipping" and lengthening the epidermis (Sharov et al., 2003) to accommodate the increase in the size of the structure that is driven by increased connective tissue production. The connective tissue of the nipple and areola region becomes enriched with immature/type III collagen and fibronectin, while SMA-labeled cells become much more prominent. The hair follicles around the nipple remain in telogen immediately after parturition and sebaceous glands expand. The areola forms as hormones in late pregnancy/parturition decline, and as mechanical strain associated with nursing commences. These stimuli activate extracellular matrix production by fibroblasts in the region below the cleft, ultimately everting the epidermis so that it forms an elevated ridge, and releasing growth factors that shift epidermal differentiation in the region. We propose that the areola allows enhanced latching by pups and the enlarged sebaceous glands at the edge of the structure provide protective lipids to the nipple and surrounding region.

Our assessment of hormone receptor expression, various nursing models that separated mechanical strain from hormone exposure, and review of the literature permits speculation as to the role of individual hormones in the process of nipple and areola expansion. The changes that occur in the late pregnant nipple are fairly consistent with the impact of the hormone relaxin (Kuenzi et al., 1995). In the pregnant nipple, *Rxfp-1* immunoreactivity or transcripts are highly expressed in the connective tissue cells that underlie the epidermis of the cleft (Kamat et al., 2004; Krajnc-Franken et al., 2004; Kuenzi and Sherwood, 1995). We also observed that phospho-CREB, the putative signal transducer of the receptor, is selectively activated in the nipple in late pregnancy (Fig. 6C). Knockout of relaxin or its receptor does not affect embryonic nipple development or the size of the virgin nipple; however, the structure does not expand during pregnancy (Kamat et al., 2004; Krajnc-Franken et al., 2004; Zhao et al., 1999). In microscopic images of a 2-day post-parturition nipple from a Rxfp1-knockout, a broad cleft was retained, suggesting that areola did not form (Kamat et al., 2004). In addition, the collagen bundles around the cleft and within the nipple itself of the Rxfp1-knockout, nipple remained very dense (Kamat et al., 2004; Krajnc-Franken et al., 2004), unlike the typical 2-day lactating samples (Fig. 3). Relaxin is recognized to both stimulate a variety of MMPs, including 1, 2, 3 and 9, and repress production of collagen I and III (Cooney et al., 2009). While the *relaxin* and *Rxfp1* knockouts indicate signaling by this hormone is necessary for areola formation, our findings

that collagen III transcripts are elevated during late pregnancy suggest that relaxin signaling alone is not sufficient for areola formation.

Estrogen alone is able to stimulate the growth of the nipple in ovariectomized rats, but not to the extent observed in normal pregnancy and lactation (Kuenzi et al., 1995). The cellular basis by which it triggers growth has not been pursued. Dermal fibroblasts have been reported to express Esr1 (Pelletier and Ren, 2004) and we found that nipple fibroblasts express elevated levels of this transcript and protein (Fig. 5&6). Also Esr1 antibody labeling was localized to the nucleus during late pregnancy consistent with canonical estrogen signaling (Fig. 6A). Evaluation of knockout mice in wound healing experiments suggests that Esr1 activates collagen production while repressing MMP expression (Takei et al., 1997; Webb et al., 2006; Gilliver et al., 2007; Gilliver and Ashcroft, 2007; Tomei et al., 2009; Campbell et al., 2010; Emmerson et al., 2013). We speculate that Esr1 may also contribute to the matrix production that drives nipple growth and areola formation.

Progesterone receptors have not been extensively studied in the skin. We observed increased levels of progesterone receptor transcripts and protein in nipple fibroblasts and nuclear labeling occurred in late pregnant nipples consistent with possible signaling. Analysis of nipple growth in ovariectomized virgin rats found that exogenous estrogen and relaxin could induce about 70% of the growth that occurred in pregnancy (Kuenzi et al., 1995), raising the possibility that there could be a role for progesterone in the growth of the structure.

A suckling mouse pup pulls the entire nipple into its mouth, inducing several forms of mechanical strain, including compression, suction, and pulling. Our manipulations that prevented nursing on nipples indicated that local suckling was necessary for areola formation (Fig. 7). Various models of mechanical strain on skin substitutes and isolated skin cells have found that it stimulated proliferation of both keratinocytes and fibroblasts, increased production of matrix transcripts such as type I collagen, fibronectin and tenascin-C, and released matrix degrading enzymes (Takei et al., 1997; Tomei et al., 2009; Webb et al., 2006). We speculate that the peak of proliferation in areola and nipple observed at the 2-day lactating point may be related in part to mechanical strain. This could activate proliferation in both the epidermis and underlying connective tissue of the nipple, as well as activate the expression of matrix genes such as *collagen III α 1 and fibronectin* (Powell et al., 2010; Sawaguchi et al., 2010).

Lactation hormones may also contribute to nipple function during nursing. Prolactin stimulates milk synthesis in the mammary epithelia, and oxytocin induces contraction in myoepithelial cells in aveoli and smooth muscle lining the lactiferous ducts facilitating milk letdown (Blass and Teicher, 1980; Neville and Morton, 2001; Svennersten-Sjaunja and Olsson, 2005). The receptors for both of these hormones are found in isolated keratinocytes and dermal fibroblasts, and ligands activate signaling in both of these cell types (Deing et al., 2013; Denda et al., 2012; Foitzik et al., 2003; Girolomoni et al., 1993). Immunoreactivity for the Prlr has been reported in the hair follicle and sebaceous gland. Additionally, the Prlr knockout mouse has longer hair cycles and produces longer, coarser hair shafts (Craven et al., 2006; Foitzik et al., 2003; Ormandy et al., 1997). We did not observe significant differences in *Prlr* transcripts in nipple fibroblasts as compared to those

from ventral skin. However we speculate that the expanded sebaceous glands, both immediately around the nipple and more generally on the ventral surface, may result from high levels of prolactin (Langan et al., 2010).

We observed that nipple fibroblasts express elevated levels of Oxtr transcripts and protein relative to ventral dermal fibroblasts (Fig. 5 and 6). Unfortunately downstream signaling from the Gprotein coupled receptor has not been extensively studied, and we were unable to probe Oxtr signaling in lactating nipples. Oxytocin has been reported to inhibit proliferation in cultured dermal fibroblasts and keratinocytes (Deing et al., 2013; Denda et al., 2012). Despite continual exposure to mechanical strain during nursing, proliferation in the keratinocytes of the nipple and areola decreased at 1-week of lactation (Fig. 2D). In contrast, the virgin nipple that was acutely nursed by pups exhibited proliferation in both the epidermis and dermis. We speculate that pulses of oxytocin generated throughout the normal lactation period may inhibit inflammation and epidermal proliferation induced by the mechanical strain of nursing.

In conclusion, the formation of the areola in the mouse results from expansion of the specialized epidermis of the nipple into the surrounding ventral skin. While the expression of nipple epidermis markers is transient in this region, the production of connective tissue expands the epidermis increasing space between hair follicles and produces a semi-glabrous region for the remainder of that animal's life.

MATERIALS AND METHODS

Mice

The K14-PTHrP transgenic line (Wysolmerski et al., 1994) was maintained as an outbred line by continual breeding against unrelated CD-1 or C57BL/6 mice. WT mice used in this study were either inbred C57BL/6 or outbred CD-1. Timed pregnant mice were generated in-house. All experiments that used animals were approved by the Indiana University IACUC and were performed in compliance with stipulations of that body.

Histology, Immunohistochemistry and Immunofluorescence

Nipples and skin were fixed in 10% buffered formalin (sodium phosphate, monobasic 4g. Sodium phosphate dibasic 6.5 g. Formaldehyde 37% 100ml and dH2O 900ml), Bouin's fixative (Sigma, St. Louis, MO) or flash frozen in OCT over night. Fixed tissues were dehydrated, embedded in paraffin, and 7μm sections were cut. Paraffin-embedded nipples were serially sectioned through the entire block. Immunohistochemistry was performed as detailed in Foley et al. (2001). The specific concentrations and modifications to the basic procedure are listed below. Alpha-smooth muscle actin (Sigma Aldrich, St. Louis, MO), filaggrin and K6 (Covance Research, Berkeley, CA), and K2e (Research Diagnostics Inc., Concord, MA) antibodies were diluted 1:3,000, 1:5,000, and 1:500, respectively, labeling required antigen retrieval in 10 mM sodium citrate under pressure in a microwave (Foley et al., 2001). BrdU immunolabeling was performed using the EMD Millipore BrdU Immunohistochemistry Kit (#2760, EMD Millipore, Billerca MA). Tissues were deparaffinized and brought to water via ethanol. Antigen retrieval was performed using

trypsin treatment (1 g crude trypsin powder, 1 g CaCl2 in 1 liter dH2O) in a 37°C water bath, followed by quenching for endogenous peroxidases using a 1:10 hydrogen peroxide stabilized in methanol solution. Blocking and antibody labeling was performed per the EMD Millipore BrdU Immunohistochemistry Kit.

For immunofluorescence, blocking solution (5% Goat or Horse serum, 1% BSA, 0.1% Triton X-100 in PBS) was applied 1 hour at room temperature, boiled in food steamer 30 mins to recover antigens. Primary antibodies were applied in 5% Goat or Horse serum, 1% BSA in PBS without Triton X-100 overnight at 4°C. Followed by 2× 5min washed in PBS, secondary antibodies (diluted in the same buffer as primary) were applied to the sections and incubated 1hr at room temperature. After 3× 5min washes in PBS, sections were then mounted with ProLong Gold antifade reagent with DAPI (P36930, Invitrogen). All microscopy images were obtained with a Nikon NiE microscope and images were analyzed with Adobe Photoshop CS6. The following primary antibodies were used: Esr1 (HC-20, Santa Cruz, SC, CA, 1:50 dilution), Relaxin receptor 1(H-160, Santa Cruz, SC, CA, 1:50 dilution), Progesterone Receptor (C-20, Santa Cruz, SC, CA, 1:50 dilution), Oxytocin Receptor (H-60, Santa Cruz, 1:50 dilution), phospho-CREB (Ser133) (06-519, EMD Millipore, Billerica, MA, 1 to 400). Secondary antibodies were used at 1:200 dilution (Jackson Immuno Research Laboratories Inc.), including Alexa-Fluor 488 or 647-conjugated goat anti-rabbit or anti-mouse IgG.

Whole mount labeling of sebaceous glands

Whole tissue sections were removed from the mouse and were prepared by washing the freshly formalin-fixed (24 hours) tissue with 50% ethanol for 10 min, removing them from their cassettes, then washing in 70% ethanol in glass scintillation bottles for 20 min on an orbiter. Tissue was then placed in Herxheimer's stain (Conn, 1969) 7 parts 100% ethanol, 2 parts 10% NaOH, 1 part ddH2O, and Sudan IV to saturation), which will stain the sebaceous glands in the skin, in 50 or 15 ml conical tubes on an orbiter for 24 hours. Stain was removed with repeated washes (20 min each) of 70% ethanol until the glands were sharply delineated. The tissue was then transferred to conical tubes full of glycerin for 24 hours. Finally, the tissue was placed on a slide, surrounded by glycerin jelly (10g gelatin, 60 ml ddH2O, and 70 ml glycerin, heated until gelatin dissolved), then coverslips were mounted on top.

BrdU incorporation and TUNEL labeling

Proliferation was assessed by BrdU incorporation through intraperitoneal injection four to five hours prior to tissue harvest. A stock solution of 20 mg/mL of BrdU in saline was prepared, and was injected 1 mg per 100 g body weight of the mice (Foley et al., 1998). Apoptosis in the nipple was evaluated on paraffin sections with the In Situ Cell Death Detection Kit (fluorescence) (Roche 11 684 795 910), according to the manufactures instructions. Nuclei were stained with Draq5 (Cell Signaling Technology, #4084S) and coverslips mounted with ProLong Gold.

Histomorphometry

All measurements and cell counts were made with Image J software. Values are expressed as the mean +/− standard deviation. Significance for the BrdU incorporation study was evaluated using ANOVA analysis and post-hoc evaluation using Tukey's test. To determine the nipple, areola, and epidermis regions, serial sectioning was done of these samples, and K2e antibody staining was done to determine where expression ended to determine the extent of the areola. Length measurement of the outer epidermis and inner epidermis was performed on 10X images of H&E stained #4 nipples from the key time points measuring the nipple and areola until the first hair follicle was encountered. Significance was determined a two-tailed Student's t-test. Area of sebaceous glands was determined with Image J on 2X images captured with a digital camera. The first 4 rows of sebaceous glands around the nipple were measured in 3 samples of the indicated time points in Figure 3 and these were compared to the average area of those from a portion of ventral skin distal from any nipples (generated from 2 samples). Significance was determined using a two-tailed Student's t-test evaluating the counts from nipples at different time points.

Picrosirius Red staining and quantification

Seven μm sections of paraffin-embedded nipple skin were deparaffinized and hydrated. The nuclei were then stained with Weigert's hematoxylin for 8 minutes, and then washed with dH2O. The sections were stained with 0.1% Picrosirius Red stain (Direct Red 80, Sigma Aldrich, 500 mL saturated aqueous solution of picric acid) for one hour, and then washed in two changes of acidified water (5 mL glacial acetic acid to 1 L dH2O). Stained sections were imaged on a Zeiss Axiophot equipped with a circular polarizer. Both blue-filtered brightfield and circular polarized images were obtained of desired areas. Image subtraction of brightfield from the circular polarized image was done suing Image J.

RNA isolation and qRTPCR

Five nipples were excised from wild-type mice, and then ground using an RNase-free tube and pestle (Kontes Pellet Pestle; Kimble Chase, Vineland NJ), and homogenized using a sonicator. RNA was extracted using the RNeasy kit (RNeasy Mini kit #74104, Qiagen, Valencia CA). RNA was then reverse transcribed into cDNA. The tissue was then analyzed for levels of gene expression of Collagen I α 2, Collagen 3 α 1, and Fibronectin relative to constitutively active GAPDH expression. Primers were designed using NCBI Nucleotide BLAST: Col1α2, sense 5'- taaacaccccagcgaagaac and antisense 5'-tgttctgagaagcacggttg, Col3α1, sense 5'- aaattctgccaccccgaact and antisense 5'- gcaccagaatctgtccacca, Fibronectin, sense 5'- gtggctgccttcaacttctc and antisense 5'- tgaatgccagtcctttaggg, GAPDH, sense 5'- catggagaaggccggggccc and antisense 5'- gatggcatggactgtggtca Esr1, sense 5'- aatgaaatgggtgcttcagg and antisense 5'- atagatcatgggcggttcag, Oxtr, sense 5'- ttcttcgtgcagatgtggag and antisense 5'- ccttcaggtaccgagcagag, Rxfp1, sense 5'- ctgcttcactgcaatggtgt and antisense 5'- cggaagtttctgcctcaaag, Prlr, sense 5'- atcattgtggccgttctctc and antisense 5'- ccagcaagtcctcacagtca, Pgr, sense 5'- ggacactggctgtggaattt and antisense 5'- cacatgacctgaccatcctg.

Fibroblast isolation

Fibroblast isolation followed the general approach developed by Lichti and colleagues (Lichti et al., 2008) Ventral skin of 6–8 weeks old female WT or K14-PTHrP were dissected and floated on 0.25% EDTA-free trypsin (Gibco) overnight at 4°C. The next day, epidermis was scraped off, minced, and enzymatically digested in a buffer solution containing 1.25mg/ml collagenase type I (Gibco), 0.5mg/ml collagenase type II (Worthington), 0.5mg/ml collagenase type IV (Sigma), and 50U/ml DNase I (Worthington) at 37°C for an hour. The preparations were then passed through blunt-end needles several times to mechanically dissociate dermis before filtering the mixture through 100μm strainer. The cells were then labeled with anti-pdgfra-PE (eBioscience). Primary fibroblast sorting was done using Aria II (BD) using CD140 antibodies (Collins et al., 2011). Flow Cytometry analysis was done using FlowJo (TreeStar Inc., version X). Re-analysis of the sorted cells indicates a 96–98% purity of CD140+ cells.

Manipulation of nursing

Nursing of naïve 8 week-old virgin females by 1 to 3 day-old pups was done over 48 hours. Groups were placed in with the dam, and were changed every 12 hours to ensure constant suckling and to avoid starvation of the pups. Artificial nursing was administered to the ventral skin distal to the nipples of the K14-PTHrP virgin mice using latex pipette bulbs coated in a powdered milk solution (Eastwood et al., 2007). The ventral skin was artificially nursed every two hours for 10 minutes in denoted regions over 48 hours. Mechanical strain was controlled by several models: (i) by removal of whole pups from dams immediately after parturition, which prevented nursing on all of the nipples but also did not continue milk, (ii) by excision of some of the nipples of dams at E15, which prevented nursing on specific nipples (not shown), and (iii) by culling the pups at parturition to 3 pups per dam, which will leave part of the 10 nipples unused (lactating non-nursed). The pups to be removed were randomly chosen and not selected based on the sex or size of the pups.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

We are ever grateful to Sue Childress for the blocking and serial sectioning of samples. This work was supported by National Institutes of Health Grant R01 AR45585, and Indiana University School of Medicine Research Enhancement Grant to JF. HJW was supported by the Medical Sciences Imogene Dahl Memorial Scholarship for thesis research. DFS is supported by a grant from the NIEHS (R01ES020866).

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Highlights

- During lactation, murine nipple specialized epidermis expands to form an areola.
- Areola formation is associated with increased extracellular matrix remodeling.
- Nipple fibroblasts express estrogen, progesterone, relaxin and oxytocin receptors.
- Areola formation requires hormones, as well as the mechanical strain of nursing.

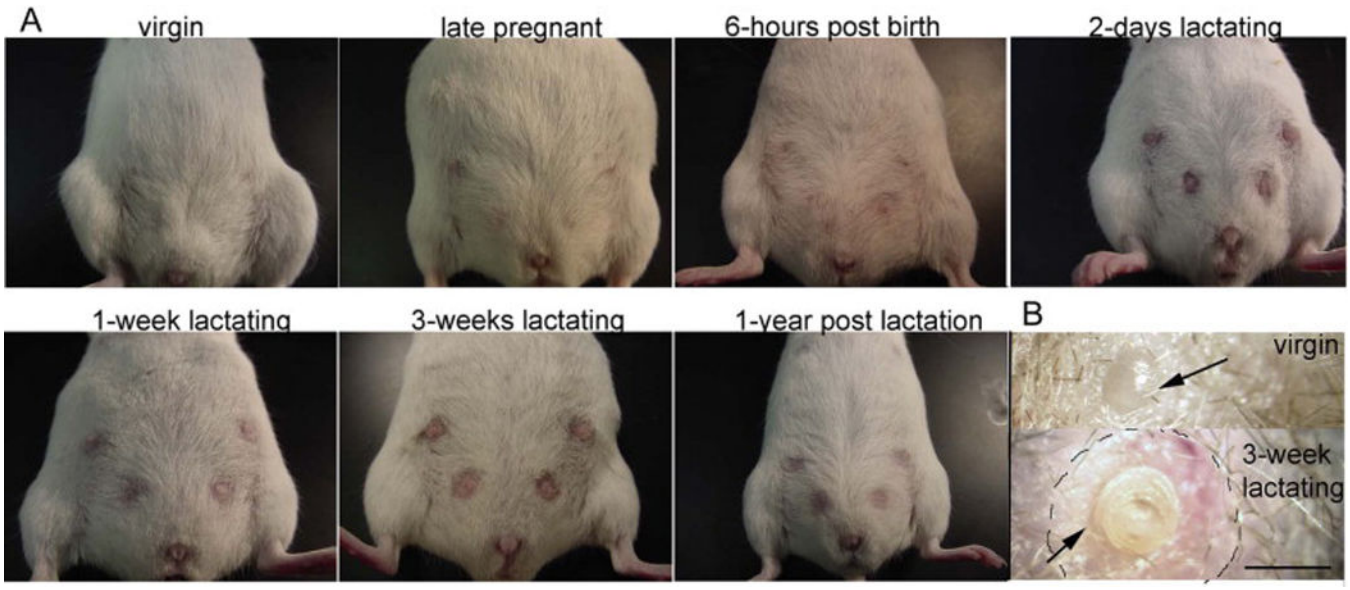

Figure 1. Nipple and areola growth and formation during pregnancy and lactation

A) Gross images of the ventral surface of mice during pregnancy as well as various time points post parturition. Note the ring of hair shaft loss is initially discernable after 2 days, reaches a maximum about 1 week after parturition, and appears to persist for the remaining life of the dam. B) Dissecting scope images of virgin and 3-week lactating nipple/areola complex where hair has been clipped around the structure to facilitate viewing. Magnification bar = 60μm in B.

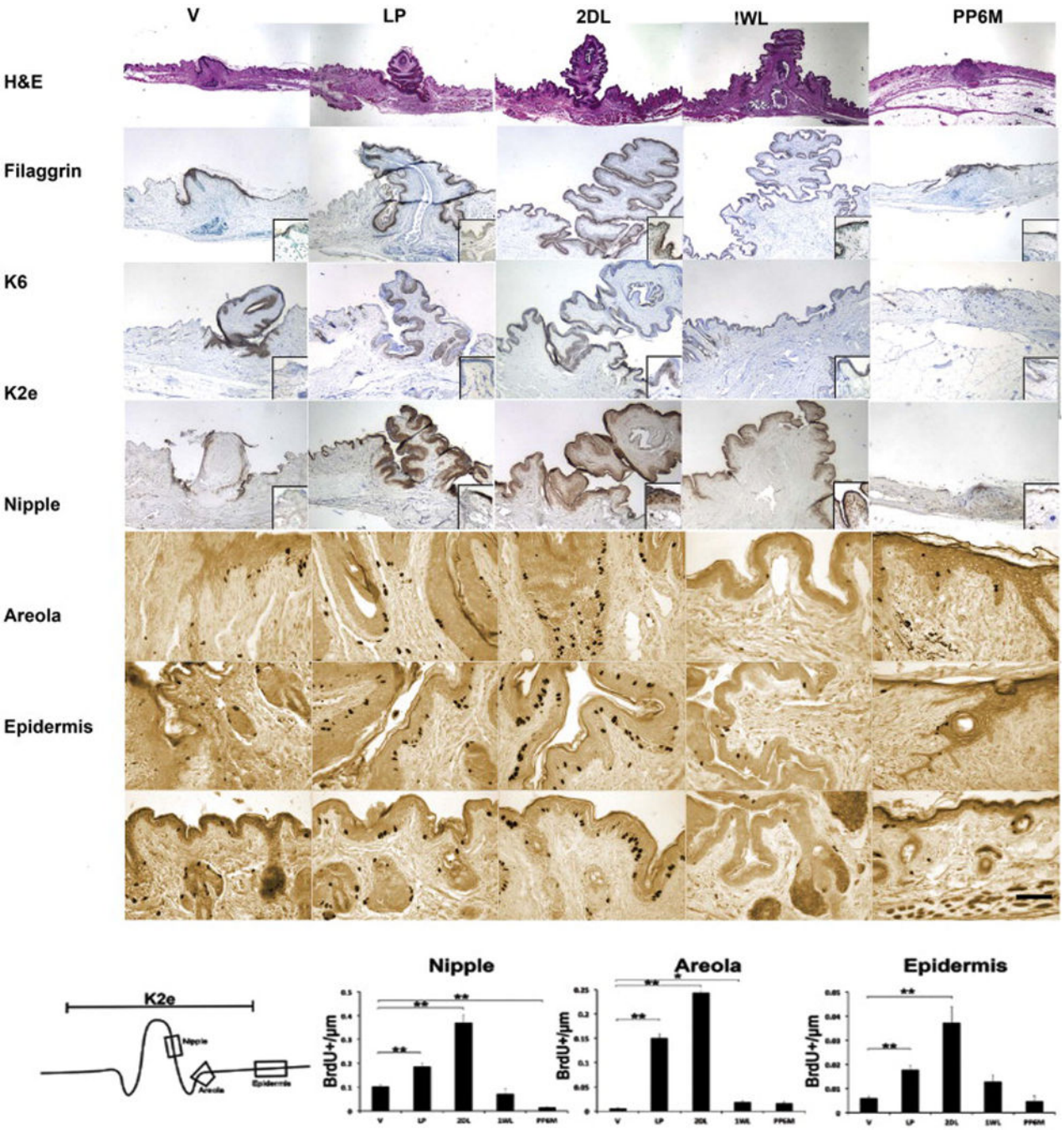

Figure 2. Epidermal changes in the areola region during pregnancy and lactation

A) Histology and immunohistochemistry of epidermal markers. Stain and antibody used are indicated on the left and the specific times on the top. The insert is a high magnification image ~0.5 mm distal from the edge of the nipple in the region which becomes areola, which shows more clearly the staining pattern for the specific marker. Magnification bar = 400 μm for the main panel of the immunohistochemistry, and 140 μm for the inserts. B) BrdU-antibody labeling for nipple, areola, and epidermal regions through pregnancy and lactation. Magnification bar = 100 μm. C) Schematic of how nipple, areola, and epidermal

regions were identified in K2e labeled slides. D) Quantification of BrdU-positive cells at the various points. Average of 3 separate #4 nipples. V: Virgin, LP: Late pregnant, 2DL: 2-day lactating, 1wk: 1-week lactating, PP6M: 6-month post lactation. Error bars indicate standard deviation. ** indicates $P<0.01$ using ANOVA.

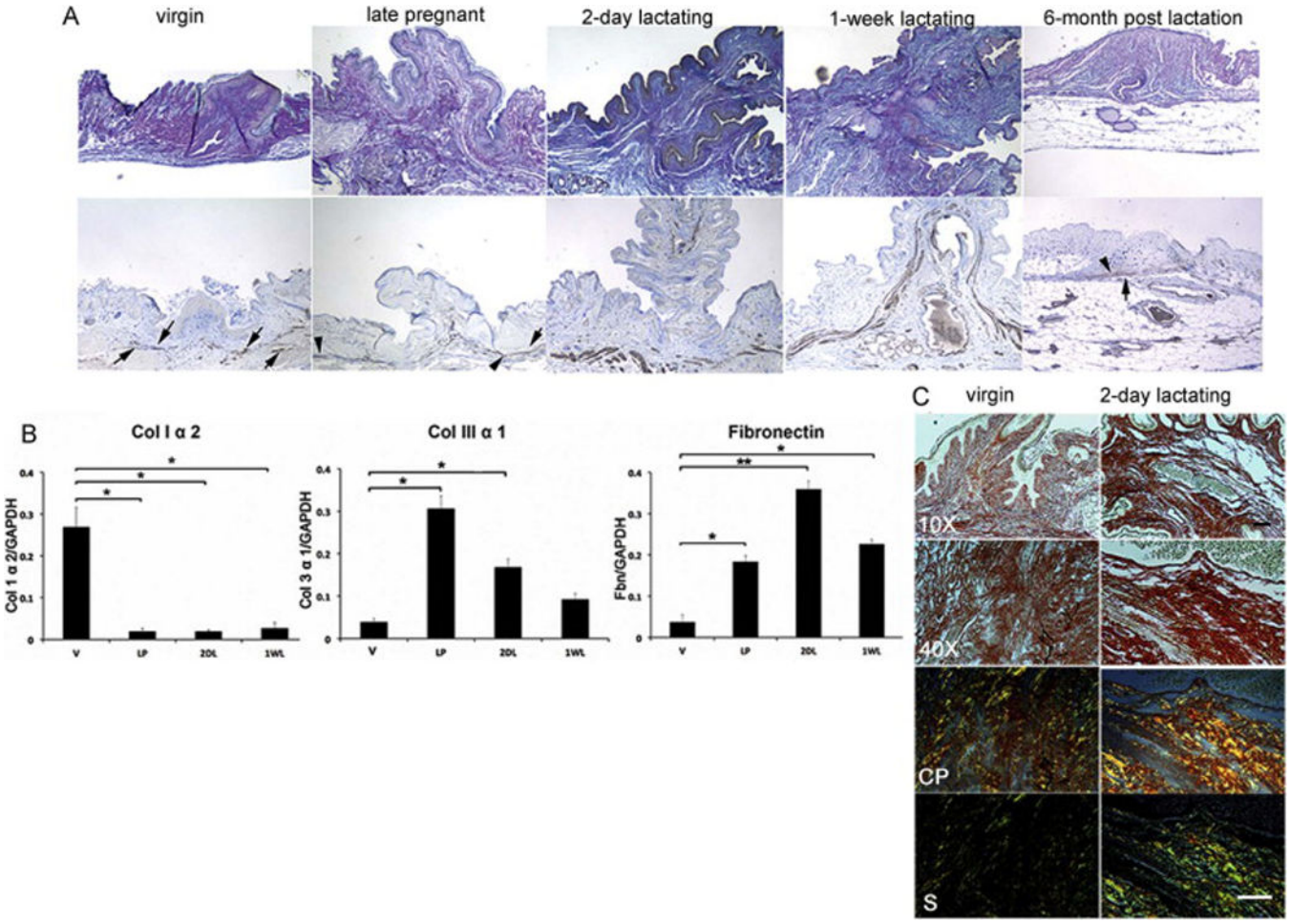

Figure 3. Connective tissue changes underlying the areola region

A) Herovici's and SMA immunohistochemistry. Type of stain or antibody are indicated on the left of the images and the reproductive status is indicated on the top. Magnification bar = 500 μm. B) qRTPCR analysis of connective tissue markers, Collagen I α 2, Collagen III α 1, and Fibronectin. V: Virgin, LP: Late pregnant, 2DL: 2-day lactating, 1WL: 1-week lactating. C) Picrosirius red stain observed with blue-filtered brightfield microscopy and circular polarized microscopy analysis of collagen types in virgin and 2-day lactating nipples. CP = circular polarized 40X image, S = image subtracted (blue-filtered bright-field 40X image from circular-polarized). Magnification bar indicates 100 μm and 50 μm in 10X and high 40X images, respectively. * and ** indicate $P<0.05$ and $P<0.01$, respectively.

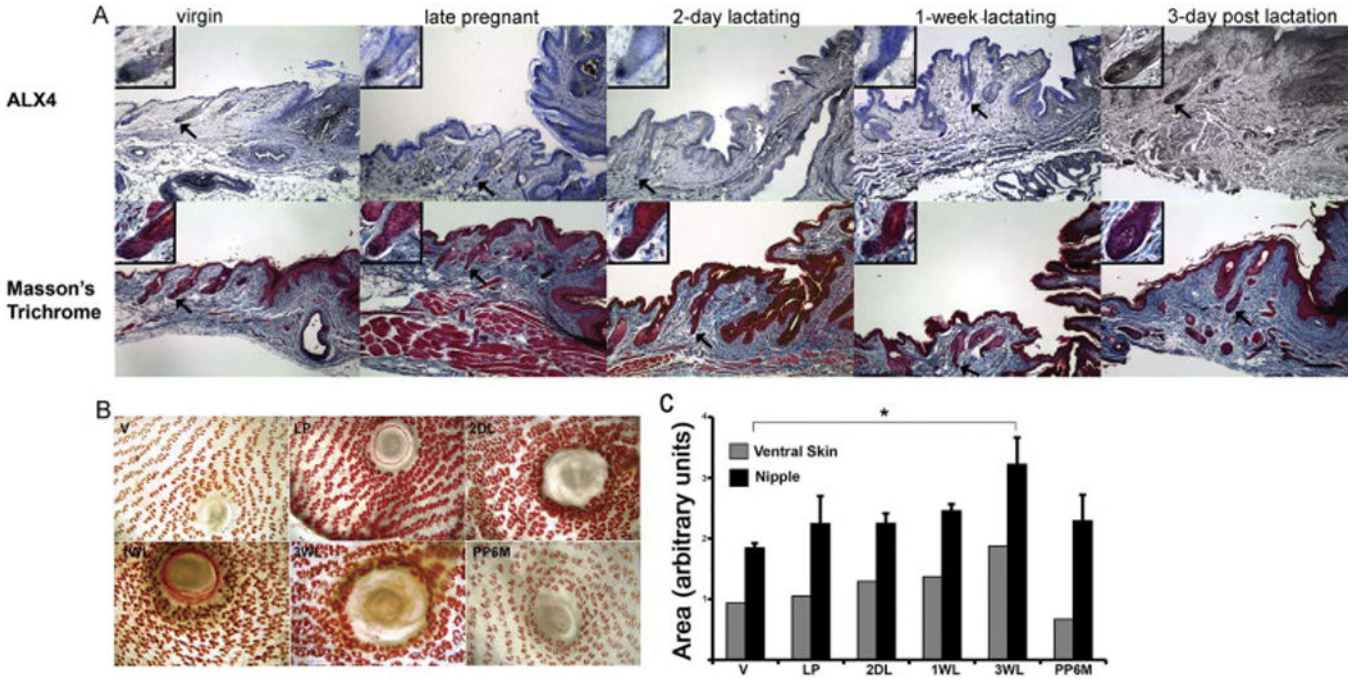

Figure 4. Changes in the pilosebaceous units in the areola region

A) The hair follicles (inset) that are in close proximity to the nipple. ALX4 immunolabeling and Masson's trichrome stain of the dermal papilla cells. Magnification bar = 100 μm for the main panel of immunohistochemistry and 25 μm for the inserts. B) Whole mount Herxheimer's stain of sebaceous glands around the nipple. C) Average area of sebaceous glands from whole mounts. Results represent average from 3 separate #3 nipples and 2 ventral skins. V: Virgin, LP: Late pregnant, 2DL: 2-day lactating, 1WL: 1-week lactating, 3WL: 3-week lactating, PP6M: 6-month post lactation. Error bars = +/− SD. * indicate $P < 0.05$ with two-tailed Student's t-test.

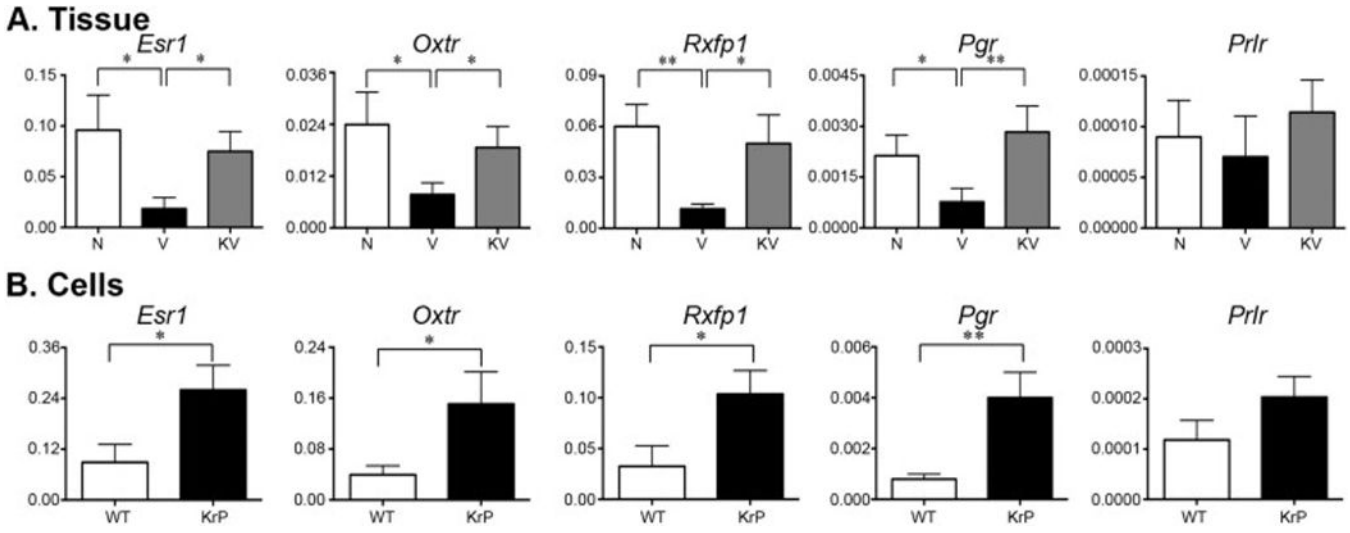

Figure 5. qRT-PCR analysis of hormone receptors expression *in-vivo* and *in-vitro*

A) qRTPCR of estrogen receptor-α (*Esr1*), oxytocin receptor (*Oxtr*), relaxin receptor (*Rxfp1*), progesterone receptor (*Pgr*), and prolactin receptor (*Prlr*) mRNA levels *in-vivo*. N, nipple of WT virgin; V, ventral skin of WT virgin; KV, ventral skin of K14-PTHrP virgin mice. B) qRTPCR of mRNA levels in sorted fibroblast cells. WT, sorted fibroblast from ventral skin of WT virgin; KrP, sorted fibroblast from ventral skin of K14-PTHrP virgin. Expression of genes was normalized to *Gapdh*. Each bar represents the average of replicates from three mice (± s.e.). * denote $P<0.05$ with unpaired t-test, ** denote $P<0.01$ with unpaired t-test.

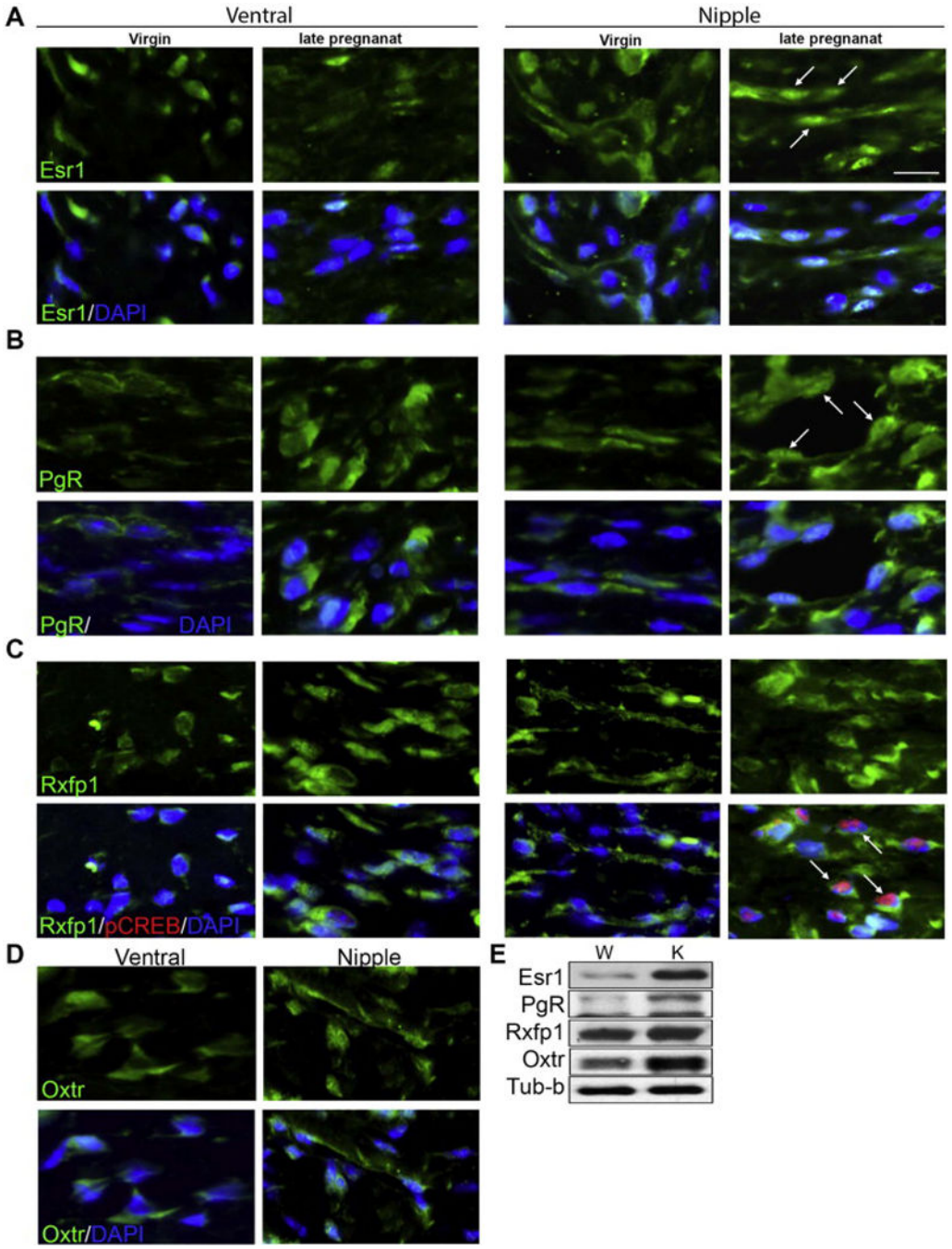

Figure 6. Hormone receptor antibody labeling

(A–D) Paraffin sections of WT ventral skin labeled with ESR1 (green, A), Pgr (green, B), Rxfp1 (green, C), phospho-CREB (red, C) and OXTR (green, D) antibodies, and counterstained with DAPI to label nuclei. Magnification bar= 50 μm. (E) Hormone receptor expression in primary fibroblasts measured by western blot. Extracts from WT and K14-PTHrP primary ventral fibroblasts probed with Esr1 (HC-20), PgR (C-20), Rxfp1 (H-160), Oxtr (H-60) antibodies. β-Tublin was used as loading control. 50 mg of total protein was loaded in each lane.

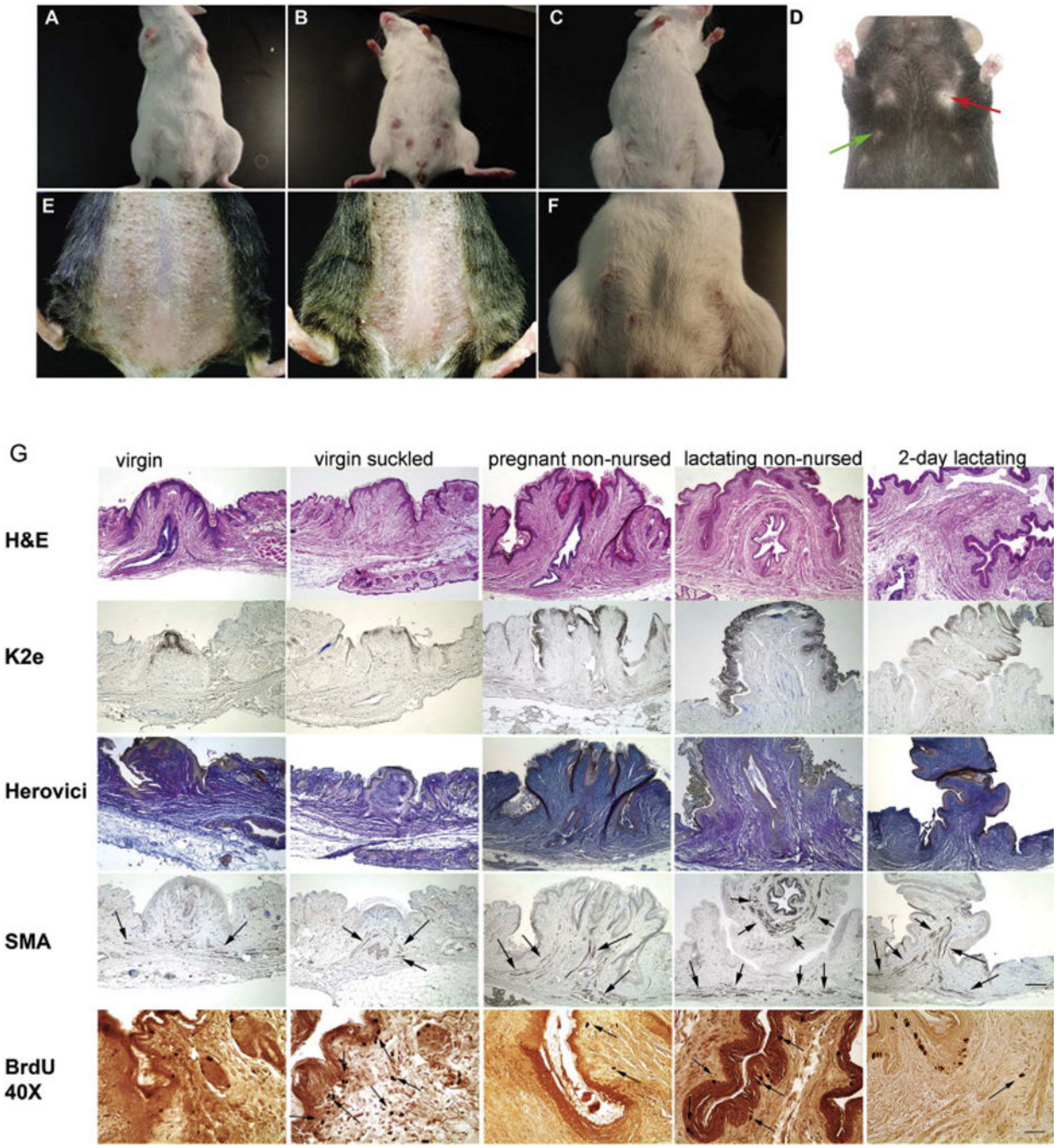

Figure 7. Manipulations that disrupt areola formation

A) Ventral skin and nipples of a dam 48 hours after pups were removed immediately after birth. B) Dam whose pups were removed after birth and then returned after 40 hours. Picture is 48 hours after pups were returned. C) Dam whose pups were removed immediately after birth and then returned 72 hours later. D) Lack of areola formation where nipple has not been nursed. Red arrow indicates #1 set of nipples that have been nursed. Green arrow indicates non-nursed #2 and 3 pairs of nipples. E) Ventral skin of K14-PTHrP pregnant (left) and early lactating (right) mice. F) Virgin mouse that has been suckled by pups for 48 hours.

G) Histology and immunohistochemical analysis of nipples from virgins that were suckled by pups for 48 hours (virgin suckled), nipples of 2 days post-delivery dam whose pups were removed at parturition (pregnant nonnursed), nipples that were not used for nursing during the first 2 days of lactation (lactating non-nursed) as compared to virgin and 2-day lactating nipples. The specific antibodies and stains used are listed to the left of the figure Magnification bars = 200μm.

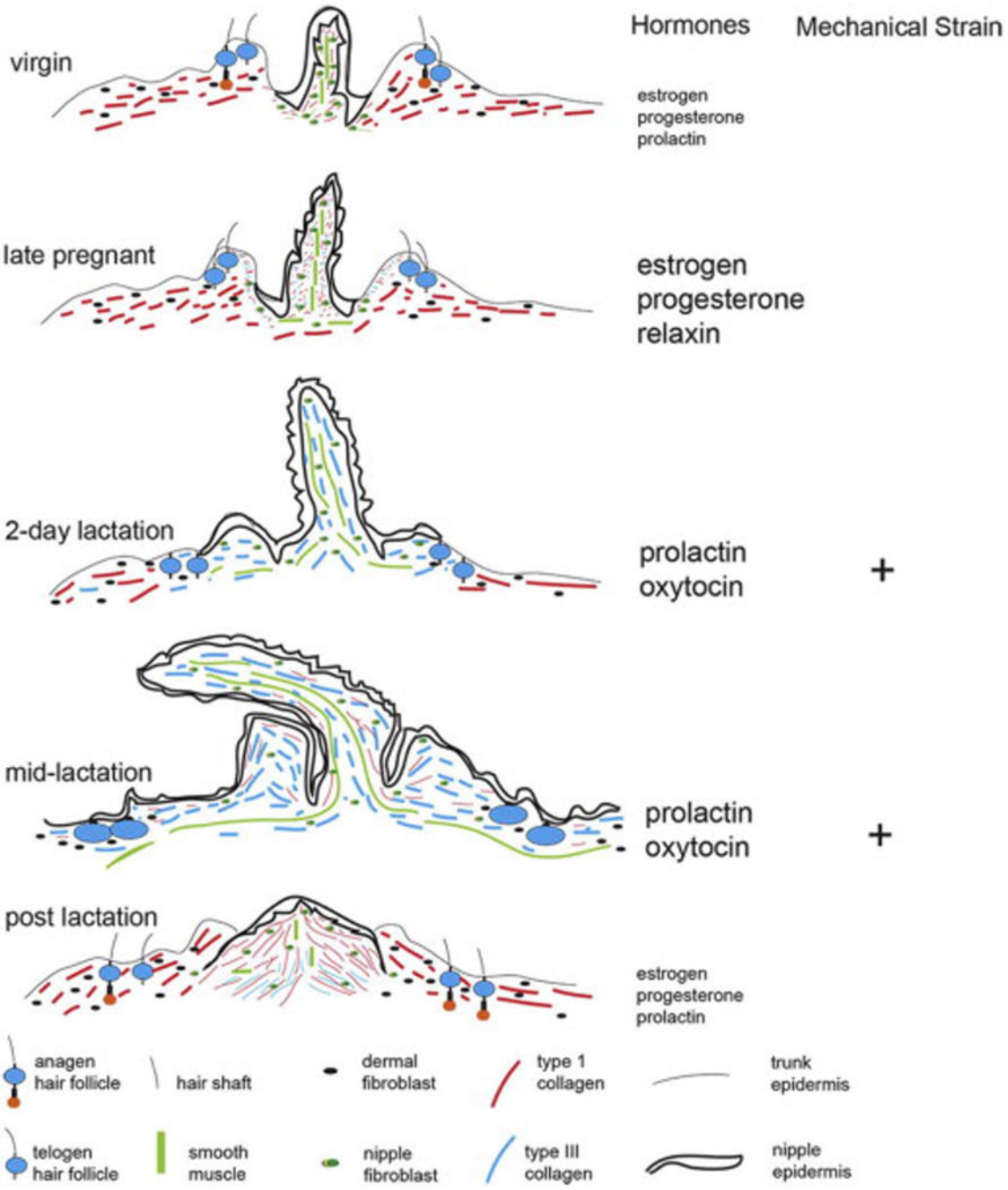

Figure 8. Proposed model of changes through pregnancy and lactation

The size of the letters reflect relative active levels of hormones at various reproductive stages. Size of pen strokes reflects relative dimensions of collagen bundles and smooth muscle bands.